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Development of a Protocol to Evaluate Neuronal Injury and Loss Following Soman-induced Seizures Using NeuN and Fluoro-Jade C

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14. ABSTRACT Previously, we examined regional neuronal damage following soman-induced seizures using Fluoro-Jade (FJ), which exhibits a marked affinity for degenerating neurons. However, with FJ alone, the degree of neuronal loss cannot be ascertained. The present study was conducted to develop a protocol to double-label a single paraffin-embedded rat brain section with both Fluoro-Jade C (FJ-C) and Neuron-specific nuclear protein (NeuN) to evaluate neuronal injury. Rats pretreated with HI-6 (125 mg/kg, i.p.) were challenged with soman (1.6 LD ₅₀ ; 180 µg/kg, s.c.) and administered atropine methyl nitrate (2.0 mg/kg, i.m.) to minimize peripheral toxic effects. All soman-exposed animals displayed prolonged convulsive behavior consistent with seizures. Brains harvested at 24 hr were paraffin-processed, sectioned, and stained with NeuN monoclonal antibody and FJ-C. The extent of neuronal injury was determined by measuring NeuN and FJ-C labeling using imaging analysis. In the piriform cortex of soman-exposed rats, FJ-C positive cells were detected in areas where NeuN immunofluorescence was lost, and pronounced necrotic lesions with depletion of NeuN staining and remnants of neuronal FJ-C fluorescence were observed. Co-localization of NeuN and FJ-C suggests that neurons are damaged, but surviving. To determine whether or not these neurons are capable of recovering, a timecourse evaluation of NeuN and FJ-C beyond 24 hr after exposure is necessary. Results indicate that double-labeling sections with NeuN and FJ-C is an effective method for assessing neuronal injury and loss.					
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Introduction

Soman (pinacolyl methylphosphono fluoridate) is a potent organophosphorous nerve agent that acts as an irreversible cholinesterase inhibitor. Acute soman poisoning results in seizures, brain damage, and death, due to accumulation of acetylcholine in the central nervous system and subsequent release of glutamate (Lallement *et al.*, 1992). Brain areas exhibiting considerable neuropathology following acute soman exposure include the piriform cortex, hippocampus, septum, entorhinal cortex, dentate gyrus, amygdala, and thalamus (Britt *et al.*, 2000; Carpentier *et al.*, 1991; Lemerrier *et al.*, 1983; and McLeod, 1985).

Previously, we examined neuronal damage in the piriform cortex of the rat following soman-induced seizures using Fluoro-Jade (FJ), an anionic fluoroscein derivative that specifically stains degenerating neuronal cell bodies and their processes, but does not stain healthy neurons (Schmued and Hopkins, 2000). The recently developed Fluoro-Jade C (FJ-C) exhibits an even greater affinity for degenerating neurons, resulting in staining with the highest resolution and contrast of the FJ dyes (Schmued *et al.*, 2005). FJ is a useful tool for detecting degenerating neurons; however, the actual degree of neuronal loss cannot be determined with FJ alone.

Neuron-specific nuclear protein (NeuN), a protein expressed in the nucleus and cell body of most neuronal cell types, has been successfully used as a neuronal marker in diagnostic histopathology (Wolf *et al.*, 1996). Healthy neurons express an intense NeuN signal, while NeuN immunoreactivity has been reported to decrease under several pathological conditions that induce neuronal cell death, such as cerebral ischemia, hypoxia, and trauma (Unal-Cevik *et al.*, 2004).

The following study was performed to develop a working protocol to double-label a single formalin-fixed, paraffin-embedded section with FJ-C for degenerating neurons and NeuN for living neurons. To test the efficacy of this double-labeling technique, we evaluated the extent of neuronal damage in the piriform cortex of the rat following acute soman exposure.

Materials and Methods

Fourteen (7 control and 7 experimental) male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 250-350 g, were used in the study. Animals were pretreated with the oxime HI-6 (125 mg/kg, i.p.) 30 min prior to soman challenge (1.6 LD₅₀ or 180 µg/kg, s.c.). One minute after soman injection, animals were treated with atropine methyl nitrate (2.0 mg/kg, i.m.) to minimize peripheral toxic effects. Control animals received an equivalent volume of vehicle, HI-6, and atropine. At 24 hr after soman exposure, animals were deeply anesthetized with sodium pentobarbital (i.p.) and transcardially perfused with 0.9% saline, followed by 10% neutral phosphate buffered formalin (NBF).

Brains were removed, post-fixed in 10% NBF for 18 hr at 4°C, and embedded in paraffin. Coronal sections (5µm thick) were cut with a microtome from bregma -3.60 mm, as shown in the Paxinos and Watson stereotaxic rat brain atlas (Paxinos and Watson, 1998), and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). After drying at room temperature for 24 hr, sections were stained sequentially with NeuN and FJ-C.

Microwave-Assisted NeuN Immunohistochemistry

Sections were deparaffinized in EZ-DeWax™ Solution (BioGenex, San Ramon, CA), hydrated to distilled water, and then incubated in 5% hydrogen peroxide for 15 min at room

temperature to suppress endogenous peroxidase activity. Following thorough washing in running tap water (5 min), sections were rinsed in distilled water, boiled for two 5-min cycles in 10 mM citric acid (Sigma-Aldrich, St Louis, MO; Lot 30H-0627) in a microwave oven (EZ Retriever® Microwave, BioGenex, San Ramon, CA), and cooled at room temperature for 20 min.

Following microwave antigen retrieval, sections were rinsed in phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO; Lot 12K8203), and incubated in 5% normal serum derived from the host for the secondary antibody for 30 min at 4°C to block tissue immunoglobulins that could react with secondary antibody. Sections were then incubated sequentially in monoclonal mouse anti-NeuN antibody (MAB377, 1:500; Chemicon, Temecula, CA) for 18 hr at 4°C, biotinylated secondary antibody (Vector, Burlingame, CA) for 1 hr at room temperature, and streptavidin conjugated with Alexa-Fluor® 594 (Molecular Probes, Eugene, OR) for 30 min at room temperature. Following incubation in Alexa-Fluor® 594, sections were rinsed in PBS and washed in distilled water. Incubating sections in FJ-C immediately after incubation in Alexa-Fluor® 594 appears to attenuate the intensity of NeuN; therefore, sections were air-dried overnight.

Fluoro-Jade C Histofluorescence

Sections were rehydrated in distilled water, transferred to a 0.0001% solution of FJ-C (Histo-Chem Inc., Jefferson, AR), dissolved in 0.1% acetic acid vehicle for 10 min (Schmued *et al.*, 2005) and then rinsed, air dehydrated, and coverslipped.

Data Analysis

Photographic documentation of double-labeling was accomplished by sequential exposures with a FITC filter for FJ-C and a Texas Red filter for NeuN using an Olympus BX61 fluorescent microscope (Olympus, Melville, NY) equipped with an Olympus DP70 digital camera. Images were merged, and quantitative evaluations of the number of FJ-C (degenerating), NeuN (living), and NeuN+FJ-C (co-localization of NeuN and FJ-C; damaged, but living) labeled neurons were performed using a computerized image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). For each rat, cells were counted in layers II and III of the piriform cortex at a magnification of 20X. A total of five counts were performed and the mean number was calculated by averaging these counts. Then for both control and experimental groups, the mean value and its standard error of the mean were calculated for 7 animals. Significant differences in the mean number of neurons labeled with FJ-C, NeuN, and NeuN+FJ-C between control and experimental groups were determined by two-way analysis of variance (ANOVA) followed by a Bonferroni test. Statistical comparisons of the average total number of neurons counted in the piriform cortex of control and soman-exposed rats were made using a two-sample t-test. In all analyses, significance was set at $p < 0.05$.

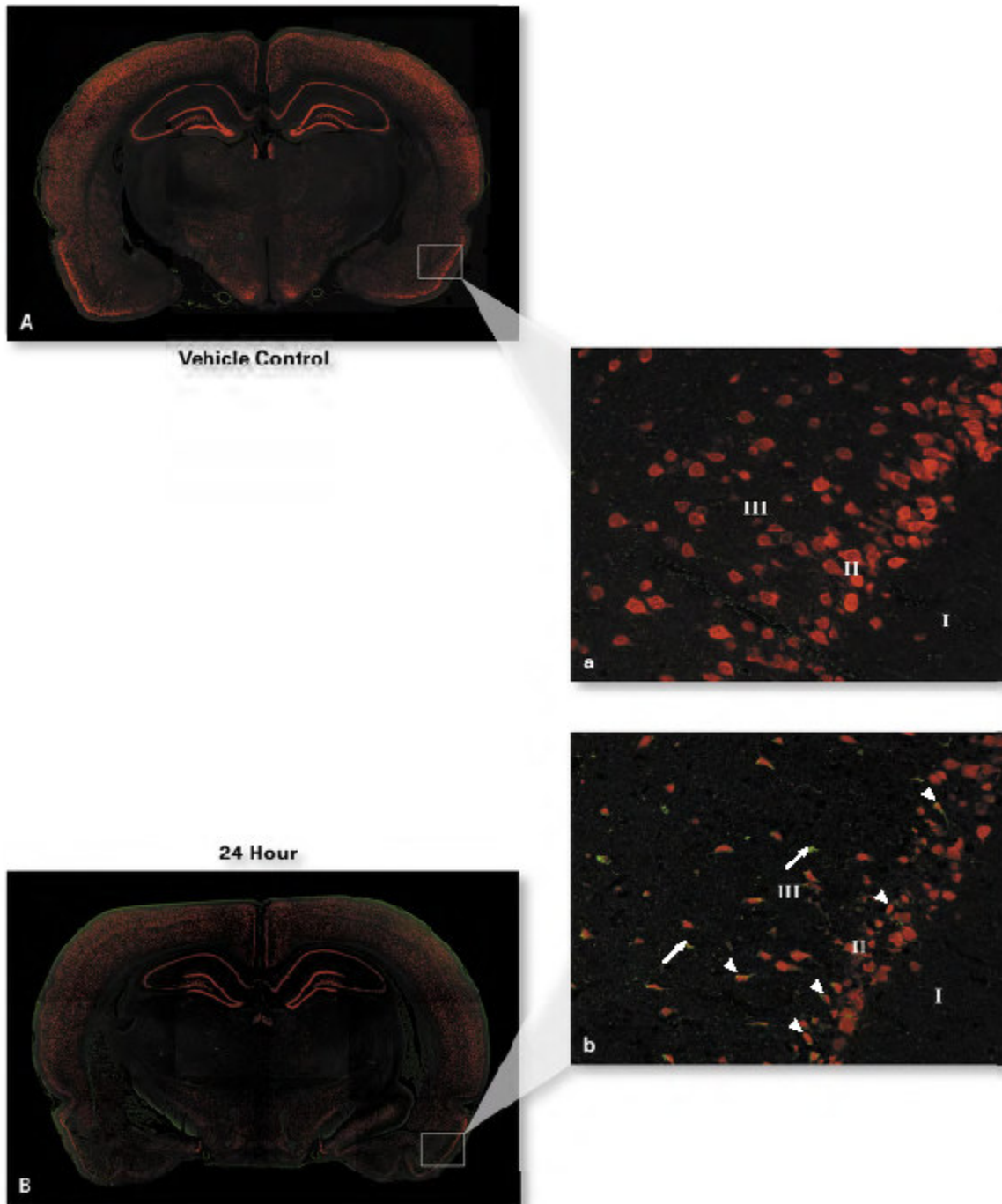


Figure 1. Vehicle control (A) and 24-hr (B) sections labeled with NeuN and FJ-C (5X). The piriform cortex of vehicle control (a) and 24-hr (b) sections was quantified (20X). At 24 hr, loss of NeuN immunoreactivity in layers II and III of the piriform cortex is evident, and surviving neurons appear shrunken. Neurons expressing NeuN+FJ-C are prominent (arrowhead) and few FJ-C positive neurons are found (arrow).

Results

Results clearly illustrate considerable loss or reduction of NeuN at 24 hr following soman exposure (Figure 1). Those neurons expressing NeuN appear shrunk in contrast to NeuN-labeled cells in control sections. In addition, a dramatic increase in the colocalization of NeuN+FJ-C at 24 hr after soman exposure is evident. Control sections were devoid of FJ-C-positive neurons, while a few were identified in the piriform cortex of 24-hr soman-exposed sections.

The average total number of neurons was significantly decreased by 58.6% (71.91 ± 1.441 vs. 42.11 ± 2.774 ; $p < 0.0001$) in layer 2 of the piriform cortex of soman-exposed rats at 24 hr (Figure 2). In layer 3 of the piriform cortex, a reduction of 52.3% (49.57 ± 4.087 vs. 25.91 ± 2.166) was significant ($p < 0.0003$) (Figure 3).

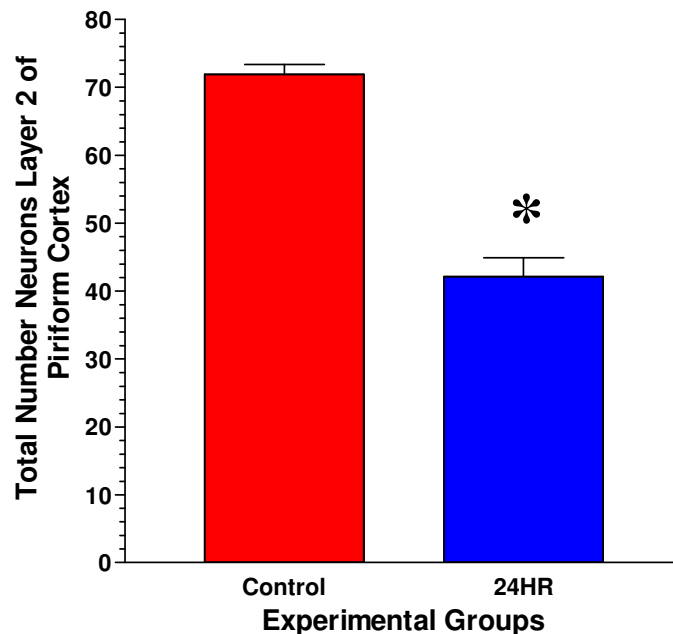


Figure 2. Quantification of the average total number of neurons in layer 2 of the piriform cortex showed significant decrease at 24 hr after acute soman poisoning. * Statistically significant.

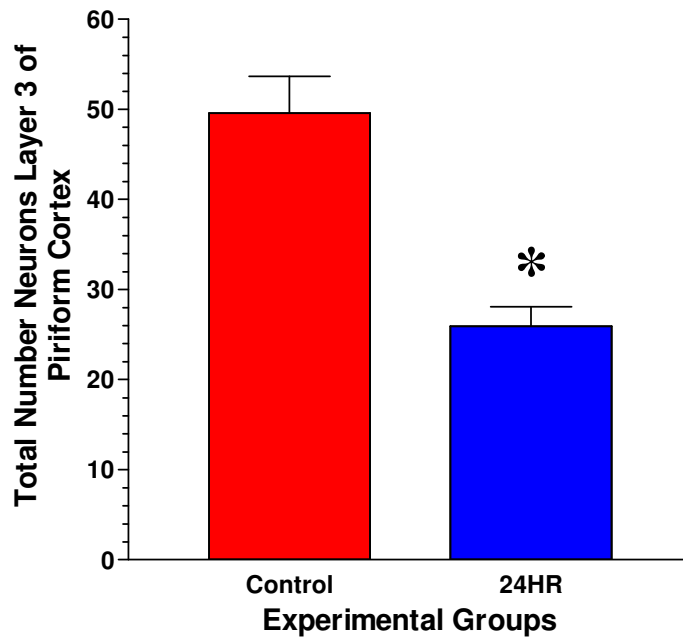


Figure 3. Quantification of the average total number of neurons in layer 3 of the piriform cortex showed significant decrease at 24 hr after acute soman poisoning. * Statistically significant.

In layer 2 of the piriform cortex of control rats, 99.7% of neurons expressed NeuN, while only a few neurons (0.28%) expressed NeuN+FJ-C (Figure 4). No FJ-C-positive cells were revealed. Soman poisoning induced a significant decrease in the number of NeuN-positive neurons in layer 2 ($p<0.001$) and a significant increase in the number of neurons expressing FJ-C+NeuN ($p<0.001$). Only 22.5% of neurons were labeled with NeuN, while 74.6% of neurons expressed NeuN+FJ-C. In addition, there was a 2.8% increase in the number of neurons expressing FJ-C; however, this increase was not statistically significant.

Similar results were found in layer 3 of the piriform cortex (Figure 5). In control rats, 99.4% of neurons were NeuN-positive, while only 0.58% of neurons expressed NeuN+FJ-C. Neurons expressing FJ-C were absent. In soman-exposed rats, 66.1% of neurons expressed NeuN+FJ-C, a significant increase from control rats ($p<0.001$). The number of NeuN-positive neurons decreased significantly to 13.5% ($p<0.001$). Although there was an increase in the number of neurons labeled with FJ-C (20.5%), this increase was not significant.

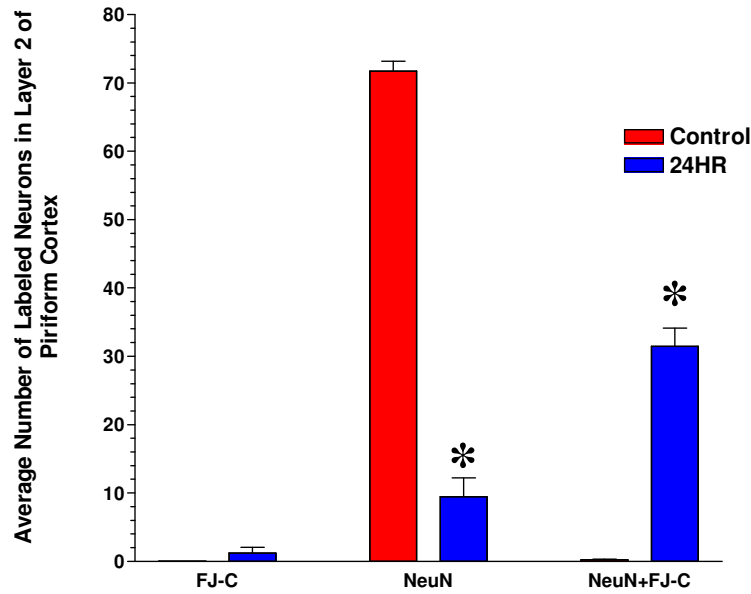


Figure 4. Quantification of the average number of FJ-C-, NeuN-, and NeuN+FJ-C-labeled neurons in layer 2 of the piriform cortex showed a significant decrease in NeuN immunoreactivity and a significant increase in NeuN+FJ-C-labeled cells at 24 hr.
* Statistically significant.

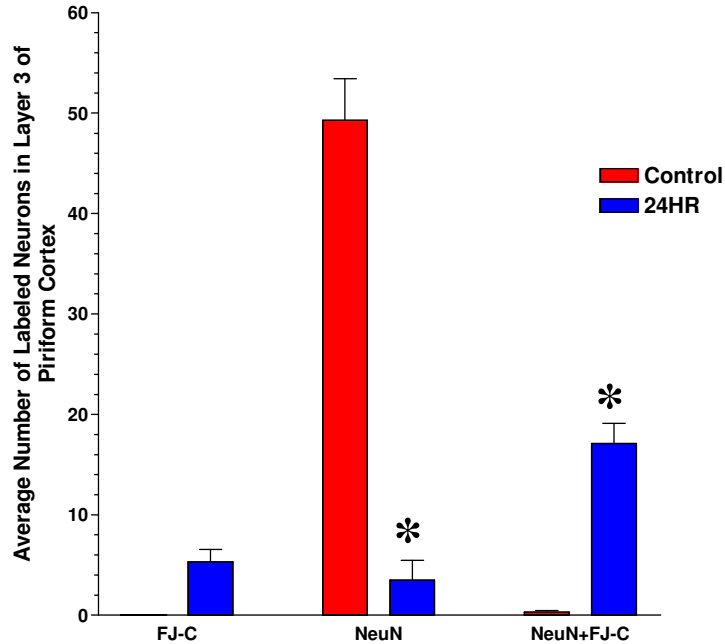


Figure 5. Quantification of the average number of FJ-C-, NeuN-, and NeuN+FJ-C-labeled neurons in layer 3 of the piriform cortex showed a significant decrease in NeuN immunoreactivity and a significant increase in NeuN+FJ-C-labeled cells at 24 hr.
* Statistically significant.

Conclusions

Double-labeling sections with NeuN and FJ-C appears to be an effective technique for evaluating the degree of neuronal injury or loss following soman-induced seizures. By 24 hr after soman exposure, the number of healthy neurons had decreased dramatically, and a significant loss of neurons was evident (Figure 2 and 3). In addition, few FJ-C positive neurons were found. These results reveal that a significant number of neurons in the piriform cortex susceptible to damage died during the first 24 hr following soman exposure. Increased co-localization of NeuN+FJ-C indicates that neurons are damaged, but surviving, suggesting that these neurons, with appropriate neuroprotective measures, may be salvaged. The hypothesis that a reversion event in which degenerating neurons revert to healthy neurons has been proposed (Collombet *et al.*, 2005). However, to determine whether or not neurons labeled with NeuN+FJ-C are capable of recovering, a time course evaluation of NeuN and FJ-C beyond 24 hr is necessary.

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APPENDIX

1. Deparaffin sections and hydrate to dH₂O.
 - a. EZ-DeWax™ Solution (BioGenex, San Ramon, CA) (2 changes, 5 min each)
 - b. Rinse thoroughly in dH₂O
2. Quench sections with 5% H₂O₂ for 15 min to eliminate endogenous peroxidase activity.

200ml of 5% H₂O₂ solution:

167ml PBS
33ml 30% H₂O₂

3. Rinse in running tap H₂O, 5 min.
4. Rinse in dH₂O, 5 min.
5. Microwave pretreatment to expose antigens:

Boil sections in 10mM citric acid, pH 6.0 (2.1 g citric acid monohydrate in 1 liter dH₂O, adjust to pH 6.0 with 2M NaOH), in microwave for 5 min. Treat 2X.

Cool for 20 min after last treatment.

6. Rinse in PBS, 5 min.
7. Incubate sections in blocking serum (5% normal serum from animals that made the secondary antibody) for 30 min at 4 °C (Vector, Burlingame, CA).

10 ml solution: 9500µl PBS
500µl (5%) normal serum

8. Incubate sections in primary antibody (1:500 dilution) for 18 hours at 4°C.

10 ml solution: 9980µl PBS
20µl primary antibody [NeuN MAB377 (Chemicon, Temecula, CA)]

9. Rinse in PBS, 5 min.
10. Incubate sections in biotinylated secondary antibody (1:200 dilution) solution at 37°C for 1 hour (Vector, Burlingame, CA).

10 ml solution: 9950µl PBS
50µl biotinylated antibody

11. Rinse in PBS, 5 min.

*******From this step on, perform as much of the protocol as possible away from light.**

12. Incubate sections in fluorescent streptavidin conjugate (1:200 dilution) solution at 37 °C for 30 min (Molecular Probes, Eugene, OR).

1 ml solution: 995µl PBS
 5µl Alexa Fluor® 594

13. Rinse in PBS, 5 min.
14. Rinse in dH₂O, 5 min.
15. Flat dry at room temperature overnight.
16. Re-hydrate with dH₂O, 5 min.
17. Incubate sections in 0.0001% Fluoro-Jade C solution for 10 min in the dark (Histo-Chem Inc., Jefferson, AR).

FJ-C stock solution (good for several months at 4 °C – discard when cloudy)

Fluoro-Jade C	50mg
Autoclaved H ₂ O	500ml

FJ-C working solution

FJ-C stock solution (0.01%)	2ml
0.1% acetic acid	198ml (198µl acetic acid in 198ml dH ₂ O)

Prepare solution in dim light.

18. 3 rinses in dH₂O, 1 min each.
19. Flat dry at room temperature.
20. Mount slides with Permount® (Fisher Scientific, Fair Lawn, NJ).